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QUALITY EVALUATION OF MONOCLONAL ANTIBODIES SUITABLE FOR IMMUNOMAGNETIC PURIFICATION OF NATIVE TAU PROTEIN

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Abstract

Tau protein plays a crucial role in the neuronal cytoskeleton stabilization. Under the pathological conditions it can be abnormally phosphorylated which leads to aggregation and formation of neurofibrillary tangles representing pathological hallmark of Alzheimer's disease (AD). For its association with neurodegenerative diseases tau protein is intensively studied in various diagnostic and therapeutic applications. Since there is no standard of tau protein involving essential post-translational modifications it is often necessary to purify it directly from cerebrospinal fluid (CSF) or blood of healthy or AD clinical signs exhibiting organism. Immunomagnetic purification based on the isolation of target protein using a specific antibody bound to magnetic carrier is the most effective tool for this purpose. High quality antibodies are the main prerequisite of successful purification, but many commercial antibodies does not comply with the challenging requirements for immunosorbent preparation. In this work, we compared 4 different anti-tau monoclonal antibodies currently

available on the market (clones HT7, BT2, 8F10, 7E5). The evaluation criteria were set along the intended use for the preparation of specific magnetic immunosorbent subsequently applicable for native tau protein purification. We evaluated the characteristics declared by producers as specificity, purity and homogeneity. We also tested the binding affinity and IgG stability during the covalent immobilization to the surface of magnetic microparticles and during the immunoprecipitation of intact tau protein or tryptic tau fragments. Results are summarized and discussed here.

Abbreviations: amino acid residues (**aa**); amyloid- β 1-42 (**A β 42**); acetonitrile (**ACN**); Alzheimer's disease (**AD**); N α -benzoyl-DL-arginine p-nitroanilide hydrochloride (**BAPNA**); bicinchoninic acid (**BCA**); bovine serum albumin (**BSA**); α -cyano-4-hydroxycinnamic acid (**CHCA**); carboxyl group (**COOH**); cerebrospinal fluid (**CSF**); carboxymethyl-cysteine (**Cys_CM**); diammonium hydrogen citrate (**DAHC**); 2,5-dihydroxybenzoic acid (**DHB**); dot-blot hybridization manifold (**DHM**); DL-dithiothreitol (**DTT**); electrochemiluminescence (**ECL**); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (**EDAC**); crystallizable fragment (**Fc**); horseradish peroxidase (**HRP**); iodoacetic acid (**IAA**); immunoglobulin G (**IgG**); immunomagnetic separation (**IMS**); immunosorbent (**IS**); linear trap quadrupole (**LTQ**); matrix-assisted laser desorption/ionization (**MALDI**); microtubule-associated proteins (**MAPs**); missed cleavages (**MC**); 2-(N-morpholino)ethanesulfonic acid sodium salt (**MES**); magnetic microparticles (**MPs**); messenger RNA (**mRNA**); mass spectrometry (**MS**); methionine sulfoxide (**MSO**); mass-to-charge ratio (**m/z**); amine group (**NH₂**); ammonium thiocyanate (**NH₄SCN**); phosphate buffer (**PB**); phosphate-buffered saline with Tween 20 (**PBS-T**); post-translational modifications (**PTM**); polyvinylidene difluoride (**PVDF**); room temperature (**RT**); sodium dodecyl sulfate – polyacrylamide gel electrophoresis (**SDS-PAGE**); N-hydroxysulfosuccinimide sodium salt (**Sulfo-NHS**);

trifluoroacetic acid (TFA); L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK); 4-chloro-1-naphthol (4CN)

Introduction

Tau proteins belong to the microtubule-associated proteins (MAPs) family operating *in vivo* as a significant regulatory element for microtubule assembly in cells by inducing the tubule formation [1]. In adult human brain, there are six major isoforms of tau generated by alternative mRNA splicing, which are phosphorylated. Since site-specific phosphorylation clearly modulates the function and intracellular localization of tau, inappropriate hyperphosphorylation caused by an imbalance in the activity of specific protein kinases or phosphatases is probably one of the key events in neurodegenerative process. Hyperphosphorylated tau protein has a lower affinity to the microtubules and drastically increased ability to self-associate and create the cytotoxic aggregates [2]. Formation of hyperphosphorylated, insoluble and filamentous tau protein is a common feature of many human neurodegenerative diseases, collectively referred to as tauopathies [3], of which the most common is Alzheimer's disease (AD) [4]. Additionally, increased phosphorylated tau and total tau levels, combined with reduced concentrations of amyloid- β 1-42 (A β 42) in cerebrospinal fluid (CSF), but not in plasma or serum, have been generally accepted as sensitive AD diagnostic markers [5].

Since phosphorylation may change some physico-chemical properties and functions of protein [6, 7] it is necessary to operate under *in vitro* studies also with the natural structures of target molecules, including post-translational modifications (PTM). On the market there is no standard of phosphorylated tau protein and *in vitro* phosphorylated recombinant molecules can differ in some way from tau protein naturally occurring in the organism. Therefore tau protein isolated directly from biological materials is useful. The isolation procedure should be

specific providing high yields and purity without the risk of structural or functional modification of isolated protein.

Purifications based on affinity chromatography represent the most powerful tool available in term of the selectivity and recovery. Batch separation with specific antibodies as affinity ligand immobilized on magnetic carriers (i. e. immunomagnetic separation, IMS) have several advantages in comparison with standard column separation procedures [8]. Separation techniques using the magnetically active microparticles are fast, gentle, easily automated and applicable in a wide range of disciplines [9]. Magnetic particles coated with antibody molecules are incubated with a protein source, after protein binding the beads are washed to remove contaminants and molecules of target protein are subsequently eluted in small volumes. Selection of high-quality antibodies represents fundamental prerequisite for effective immunopurification of target antigen. Antibodies with the desired specificity are usually provided by several different manufacturers and companies, but the quality of the antibodies and their suitability for designate application can significantly differ.

In this paper, we evaluated four various commercial monoclonal antibodies with the specificity against all six isoforms ensuring total tau protein isolation. The specificity of monoclonal antibody clone 8F10 is directed against the C-terminus of microtubule binding-domain of tau protein molecule (epitope: aa 428-437), epitopes of antibody clones HT7 and 7E5 are included in the N-terminal projection domain and epitope of antibody clone BT2 is located between these two domains (see Tab. 1). The specific immunoreactivity with tau protein and affinity of the antibodies was verified by dot-blot analysis [10]. Consequently immobilization of the antibodies onto magnetic microparticles with carboxyl function groups was performed and these immunosorbents (IS) were applied for immunomagnetic separation of recombinant tau protein or tryptic peptide fragments of tau. The efficiency of antibodies immobilization was evaluated by simple BCA test. Elution fractions collected during the IMS

experiments were analyzed by dot-blot and mass spectrometry (MS). The main goal of this work was to select IgG molecules suitable for intended tau protein purification from liquid biological materials such as CSF or serum. The scheme of the whole experiment is mentioned on Fig. 1.

Figure 1

Experimental

Materials and Equipment

Four types of monoclonal anti-tau antibodies were compared (see Tab. 1): clones HT7, BT2 were obtained from Pierce (Rockford, IL, USA), clones 8F10, 7E5 from AJ Roboscreen GmbH (Leipzig, Germany). Recombinant tau protein (human 2N4R variant, isoform 441) was supplied by rPeptide (Bogart, GA, USA). SeraMag Speed Beads Magnetic Carboxylate-Modified Particles (0.816 μm) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Tau monoclonal antibody (Tau 5) applied as detection antibody in quantitative dot-blot analysis was a gift from Francisco Garcia-Sierra (Center of Research and Advanced Studies of the National Polytechnic Institute, Mexico). Polyclonal rabbit anti-mouse IgG/HRP antibodies were obtained from Dako (Glostrup, Denmark). Bovine serum albumin (BSA), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.22.2, 12,700 IU/mg solid), DL-dithiothreitol (DTT), iodoacetic acid (IAA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), N α -benzoyl-DL-arginine p-nitroanilide hydrochloride (BAPNA), 2-(N-morpholino)ethanesulfonic acid sodium salt (MES), acetonitrile (ACN), benzamidine and diammonium hydrogen citrate (DAHC) were produced by Sigma–Aldrich (St. Louis, MO, USA). N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS) and trifluoroacetic acid (TFA) were obtained from Fluka (Buchs, Switzerland). The Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL, USA).

PVDF membrane (Immuno-Blot PVDF Membrane, 0.2 μm), Clarity Western ECL substrate and Immun-Blot Opti-4CN Colorimetric kit were acquired from Bio-Rad (Hercules, CA, USA). Poros Oligo R3 reversed-phase material was purchased from Life Technologies (Carlsbad, CA, USA). Matrices for MS analysis, α -cyano-4-hydroxycinnamic acid (CHCA) and 2, 5-dihydroxybenzoic acid (DHB) were from LaserBio Labs (Sophia-Antipolis, France). All other chemicals were of reagent grade.

Magnetic separator was acquired from Dynal (Carlsbad, CA, USA). Amicon[®] Ultra 0.5 mL filters were purchased from Merck Millipore (Billerica, MA, USA). GELoader tips were from Eppendorf (Hamburg, Germany). MS instrument MALDI LTQ Orbitrap XL was obtained from Thermo Scientific (Waltham, MA, USA), SpeedVac RVC 2-18 from Christ (Osterode am Harz, Germany) was connected with vacuum pump KNF Neuberger (Freiburg, Germany). Dot-blot DHM-96 unit manifold was purchased from Scie-Plast (Cambridge, UK).

Table 1

SDS-PAGE analysis

1 μg of each tested antibody (Tab. 1)/tau protein sample was mixed with Laemmli sample buffer/Tricine sample buffer with or without β -mercaptoethanol (1:1), boiled at 100°C for 3 min and then loaded onto a 0.75 mm Tris-glycine gel (10% [w/v] separating gel) according to Laemmli [11]/Tris-tricine gel (16.5% T, 3% C [w/v] separating gel) according to Schägger [12]. Electrophoresis was performed using a Mini-Protean system (Bio-Rad, Hercules, CA, USA) at 180 V in Tris-glycine-SDS/30 and 100 V in Tris-tricine-SDS running buffer. Proteins in the gels were visualized by a silver staining method [13] and pictures were taken with a digital camera Nikon Coolpix 5000 (Nikon, Tokyo, Japan).

Immobilization of antibodies to the magnetic microparticles

20 µg of monoclonal anti-tau antibodies (Tab. 1) were covalently bound to 0.5 mg of SeraMag magnetic microparticles (MPs) by two-step carbodiimide method using EDAC (120 mM)/Sulfo-NHS (20 mM) [14, 15] in 50 mM MES buffer pH 6.0 overnight at 4°C and under mild stirring. Prepared immunosorbents were stored in 0.1 M phosphate buffer pH 7.0 containing 0.05% sodium azide at 4°C. Immobilization efficiency was evaluated from the difference of antibody concentration in solution before and after immobilization determined by BCA test. BCA assay was performed in 96-well plate arrangement according to manufacturer's recommendations.

Proteolytic digestion of tau protein

Trypsin specific magnetic carrier was prepared using the TPCK-treated trypsin (9 mg) pre-incubated for 10 min at room temperature (RT) with benzamidine (2.5 mM) and consequently mixed with SeraMag MPs (3 mg). Formation of the covalent bonds was performed by one-step carbodiimide method using EDAC (120 mM)/Sulfo-NHS (20 mM) [14, 15] in 0.1 M phosphate buffer pH 7.3 overnight at RT under mild stirring. Activity of immobilized TCPK-trypsin was determined in 96-well plate by hydrolysis of chromogenic substrate BApNA [16]. Biofunctionalised beads were stored in 0.1 M phosphate buffer pH 7.0 containing reversible enzyme inhibitor benzamidine (2.5 mM) at 4°C.

Protein to be digested (recombinant tau protein, isoform 441) was firstly unfolded by reductive alkylation using DTT and IAA [17] in 50 mM ammonium bicarbonate solution and subsequently digested by TPCK-trypsin immobilized on magnetic particles in molar ratio E : S of 1 : 20 for 2 h at 37°C under mild stirring. The efficiency of digestion was verified by Tricin/SDS-PAGE electrophoresis [12] and MALDI-MS analysis. The mixture of tryptic tau fragments was also used for immunomagnetic separation.

Immunomagnetic separation

5µg of tau protein (recombinant, isoform 441) or mixture of tryptic tau fragments was added to the immunosorbents (0.5 mg) prepared by immobilization of four various monoclonal anti-tau antibodies and pre-washed with 0.1 M phosphate buffer pH 7.0. Incubation for 4 h, at RT under stirring followed. Intensive washing was carried out by 0.1 M phosphate buffer pH 7.0 (containing 0.2 M /1 M NaCl), 0.01 M phosphate buffer pH 7.0 and ultra-pure water. Finally elution of immunocaptured tau molecules or tryptic tau fragments was performed three times for 20 min by 0.2 ml of 0.05% TFA at RT and under stirring. Pooled eluted fractions were dried in speed-vac and analyzed by dot-blot analysis (proteins) or by MS-analysis (peptides).

Experiment with tryptic tau fragments was repeated also with incubation (10 min, RT, under stirring) of immunosorbents after immunocomplex formation (4 h incubation) with 8M urea to minimize unspecific protein-protein interactions.

Dot-blot analysis

Dot-blot experiment was performed according to already published protocol [10] with slight modifications. Analyzed samples dissolved in 100 µl of 0.1 M phosphate buffer pH 7.0 (PB buffer) were spotted on equilibrated PVDF membrane and washed by 100 µl of PBS-T buffer. Then membrane was blocked using 5% BSA in PBS-T for 60 min and incubation with primary antibody diluted in the ratio 1:30000/1:10000 for 60 min was performed. Washing with PBS-T and incubation for 60 min with secondary antibody (anti-mouse IgG/HRP) diluted in the ratio 1:10000/1:1000 followed. Finally, washing with PBS-T and chemiluminescence detection by Clarity western ECL substrate/colorimetric detection using Opti-4CN kit according to the manufacturer's instructions was carried out.

For affinity evaluation of monoclonal anti-tau antibodies (Tab. 1) recombinant tau-441 protein (0.03 µg per spot) was applied and chaotropic step using 0 - 2 M NH₄SCN was included in addition [10]. ChemiDoc™ MP Imaging System with Image Lab™ Software (Bio-Rad, Hercules, CA, USA) was applied for documentation and dot-blot analysis.

Mass spectrometry analysis

Chromatographic reversed-phase (Poros Oligo R3) microcolumns used for desalting and concentration of peptides were prepared using GELoader tips as previously described [18]. Dried tau peptide fragments were dissolved in 0.1% TFA and applied onto Poros Oligo R3 microcolumns using gentle air pressure. The columns were washed with 15 µl of 0.1% TFA and retained peptides were eluted directly onto MALDI-targets by 1 µl of CHCA solution (5 mg/ml of 70% ACN/0.1% TFA + 2 mM DAHC). A MALDI-Orbitrap MS instrument was used for measurement in positive mode with pulsed nitrogen laser operating at 337 nm.

Results and discussion

Two conditions has to be met for successful immunomagnetic purification: i) reactivity with biospecific paired molecules retained even after the covalent binding of ligand (IgG) to the solid phase, and ii) the interaction between the ligand and target molecule must be reversible to allow the target molecules to be released in an active form. IgG molecules providing the desired specificity and appropriate affinity are the most suitable ligand for immunosorbent preparation. Monoclonal antibodies compared with polyclonal are more desirable due to their lack of variability which allows preparing robust and more uniform carrier [19]. In this work we evaluated and compared four mouse monoclonal antibodies from

two suppliers with specificity for tau protein differing in their isotype, epitope reactivity or used immunogen (see Tab. 1).

The rating quality of antibodies, it means purity, affinity, specificity and no cross-reactivity, should be considered prior to covalent immobilization to the solid phase. We used SDS-PAGE and dot-blot analysis for qualitative evaluation of all tested antibodies. As can be seen in Fig. 2, all antibodies are pure, homogeneous and non-fragmented. After the reduction and alkylation of disulfide bonds by β -mercaptoethanol (positions 6-9), the differences in molecular weights corresponding to the light and heavy chains are observed. These discrepancies can be due to the different carbohydrate content or isotype affiliation.

Figure 2

Dot-blot results (Fig. 3A) confirmed the specific reactivity of all antibodies with recombinant tau protein isoform 441, but BT2 antibodies in comparison with others show lower level of signal, using 0.025 μ g of antigen the signal was almost zero (spot No. 2). Moreover, no antibodies showed cross-reactivity with BSA, which was spotted on the membrane in position 3 as a negative control. Dot-blot experiment supplemented by chaotropic step with different concentrations of ammonium thiocyanate (NH_4SCN) in the range 0 - 2 M was applied for determination of affinity index of tested antibodies (Fig. 3B) [10]. Affinity index is expressed as the molarity of chaotropic reagent causing 50% reduction of the initial signal [20]. Clones BT2, 8F10 and HT7 showed similar value of the affinity index around 0.75 mol/L, the signal corresponding to the amount of immunocomplex decreased significantly with increasing concentration of ammonium thiocyanate. The highest affinity index (1.3 mol/L) was observed for 7E5 clone, which indicates its strongest interaction with recombinant Tau protein.

Figure 3

The type of covalent bonds and the orientation of affinity ligand are very important when designing an affinity chromatography in a preparative mode. Antibody immobilization onto solid surface has been studied extensively for a number of applications [21]. There are a number of immobilization strategies, including covalent coupling, adsorption or affinity binding, each of which has its benefits and drawbacks. Covalent coupling is one of the most common ways of attaching an affinity ligand to solid support material and we use it when a very active and stable microsphere reagent is required [22]. There is a wide range of coupling chemistries available when considering covalent immobilization methods. Random covalent immobilization methods generally link antibodies to the solid support via their free amine groups [23]. Anti-tau IgG molecules can be attached to the surface of carrier through the widely occurred COOH or NH₂ groups or by specific functional groups, e.g. generated by oxidation of terminal galactoses of glycosidic carbohydrate chains localized in Fc part of antibody molecule. When immobilizing an affinity ligand, care must be taken to the steric accessibility of sites intended for biospecific interaction. Also the binding affinity of ligand to the target antigen has to be maintained [19].

In our work we used common carbodiimide method (EDAC) for crosslinking of carboxyl groups placed on the magnetic beads with amino groups of antibody molecules. Sulfo-NHS was added for improving the binding efficiency [14]. Optimization of the coating protocol (amounts and ratios of all reagents, 1-step/2-step procedure, and incubation time) should lead to the high-quality immunosorbent. In our experiment 20 µg of each monoclonal anti-tau protein antibody was bound on 0.5 mg of SeraMag beads. Binding efficiency was determined by SDS-PAGE (data not shown) and simple BCA method estimating protein content in the solution before and after the immobilization. The results in Fig. 4 show, that all antibodies were bound to SeraMag MPs in quite a large amount, the lowest efficiency (65 %)

was obtained with BT2 antibodies, the rest of the antibodies was bound in quantity greater than 80 %.

Figure 4

Prepared magnetic immunosorbents comprising MPs with specific antibodies were applied for immunomagnetic purification of recombinant tau protein isoform 441 (5 µg). Binding capacity of the immunosorbents was determined by dot-blot analysis of captured and consequently eluted tau protein. Dried binding fractions, first washing fractions and pooled eluted fractions from IMS experiments performed with the whole tau protein were dissolved in 100 µl of PB buffer and spotted on equilibrated PVDF membrane, as well as samples for calibration curve construction (0.0625 - 1 µg of recombinant Tau 441/100 µl of PB buffer) and negative control (1 µg of BSA/100 µl of PB buffer). High-sensitive chemiluminescence detection was applied in this case. The results of dot-blot quantification (Fig. 5) show that all immunosorbents are able to effectively bind tau protein and could be used for immunomagnetic purification. The highest binding capacity was observed using immunosorbents with 7E5 and 8F10 antibodies.

Figure 5

We tried to repeat immunomagnetic separation experiment also with the mixture of tau protein fragments generated by tryptic digestion. All antibodies have defined specific epitope corresponding to the amino acid sequence in tau protein structure to which antibodies are specifically bound. The goal was to verify the immunoreactivity of antibodies also at the peptide level, because *in vivo* tau protein can also be found in truncated forms. In recent years, besides the well documented role of abnormal phosphorylation of tau, other modifications such as proteolytic cleavage at the C-terminus of the molecule have been linked also to the

pathogenesis of AD [24]. Mass spectrometry (MS) was used for detection of peptide samples. Theoretical peptide fragments of tau protein 441 obtained by digestion with trypsin, which include within the sequence epitopes of individual antibodies and can be expected in the MS spectra, are listed in Tab. 2.

Table 2

For tau protein fragmentation we applied highly specific TPCK-trypsin immobilized on MPs. When trypsin is immobilized, it can be easily removed from the final peptide mixture using a magnetic separator so the sample is free of trypsin contaminants, moreover we can use it repeatedly and minimum of autolytic products are generated. The final activity of immobilized trypsin determined by synthetic substrate BApNA was 996 IU/mg of MPs. Proteolytic digestion of tau protein was carried out for 2 hours at 37°C and with a molar enzyme-to-substrate ratio of 1:20. The reduction and alkylation of disulfide bonds using DTT and IAA respectively were performed prior the digestion which helps to denature proteins, making their proteolytic sites more accessible for proteolysis. The efficiency of tryptic digestion was confirmed by Tris–tricine–SDS-PAGE and MALDI-MS analysis. The results of Tris–tricine–SDS-PAGE (Fig. 6B) demonstrated complete tau protein digestion. In MS spectrum (Fig. 6A) we can see specific tau fragments including epitopes but also some non-specific fragments are present.

Figure 6

Mixture of peptide fragments obtained by tryptic digestion of tau protein (5 µg) was applied to individual anti-tau immunosorbents. Only peptides including specific epitope should be captured by antibody, other peptides are washed away. Captured peptides are

subsequently eluted with acid pH and analysed by MS. Two experiments with or without 8M urea incubation step were performed and compared.

Figure 7

The results of MS analysis (Fig. 7) show the most effective and specific immunocapturing peptide reaction using immunosorbent with 7E5 antibodies, specific peak $m/z = 1423.74$ dominates in spectrum of elution fraction collected in IMS experiment without urea incubation (Fig. 7A). Residues of this peptide are visible also in the elution fraction after treatment with 8M urea (Fig. 7B) which confirms a higher affinity of these antibodies to specific peptide fragment. In other elution fractions specific peptide fragments are also detected but with lower intensity and together with non-specific peptides. In the case of 8F10 peptide including specific epitope wasn't observed. 8M urea was applied to remove peptide fragments non-specifically adsorbed to the carrier for increasing the purity of the elution fractions, which should contain only the specific epitope-containing peptide fragments. As seen on Fig. 7B some non-specific fragments resist the urea effect and they are still observed in spectra, on the contrary weaker specific interactions were suppressed which was demonstrated by reduction (BT2, 7E5) or disappearance of specific fragments (HT7). All obtained results are summarized in Tab. 3.

Table 3

Conclusion

Our results show that mouse monoclonal antibody, specific clone 7E5, directed against the N-terminal domain of tau protein (aa 156-165), is the most suitable from tested anti-tau IgG molecules for immunosorbent preparation and subsequent immunomagnetic purification of tau protein. It shows the strongest immunoreactivity with both whole tau protein as well as

the specific peptide fragments. As producer declared these IgG molecules are phospho-insensitive. Therefore, clone 7E5 is a good candidate for immunomagnetic purification of all forms, including hyperphosphorylated, of native tau protein from complex biological material such as cerebrospinal fluid or serum.

Acknowledgement

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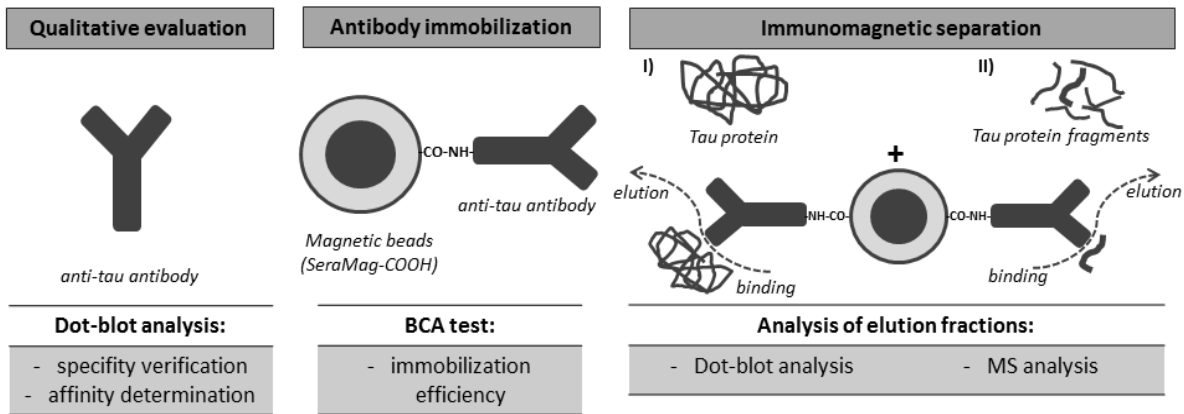


Figure 1: Schematic representation of the comparative experiment.

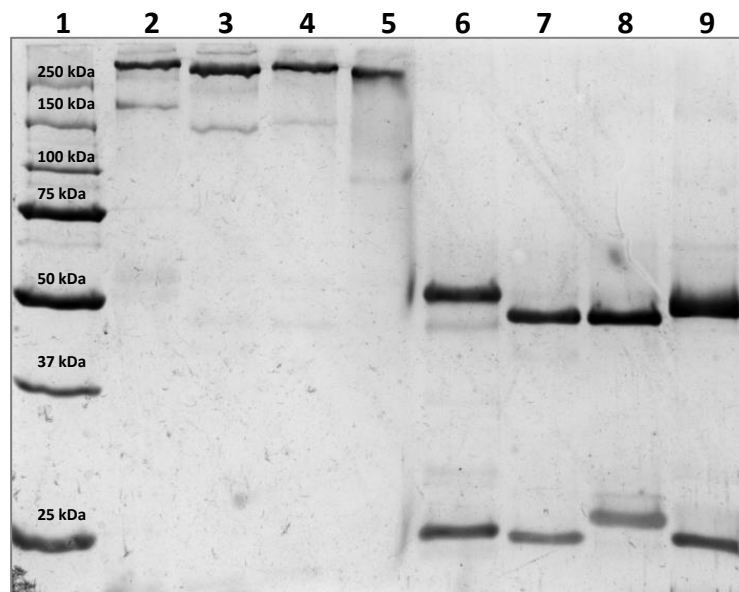


Figure 2: SDS-PAGE analysis of mouse monoclonal anti-tau protein antibodies followed by silver staining; lanes: 1 – marker of molecular weights (10-250 kDa), 2 and 6 – clone HT7, 3 and 7 – clone BT2, 4 and 8 – clone 8F10, 5 and 9 – clone 7E5; positions 2 – 5 are without β -mercaptoethanol, positions 6 – 9 are with β -mercaptoethanol.

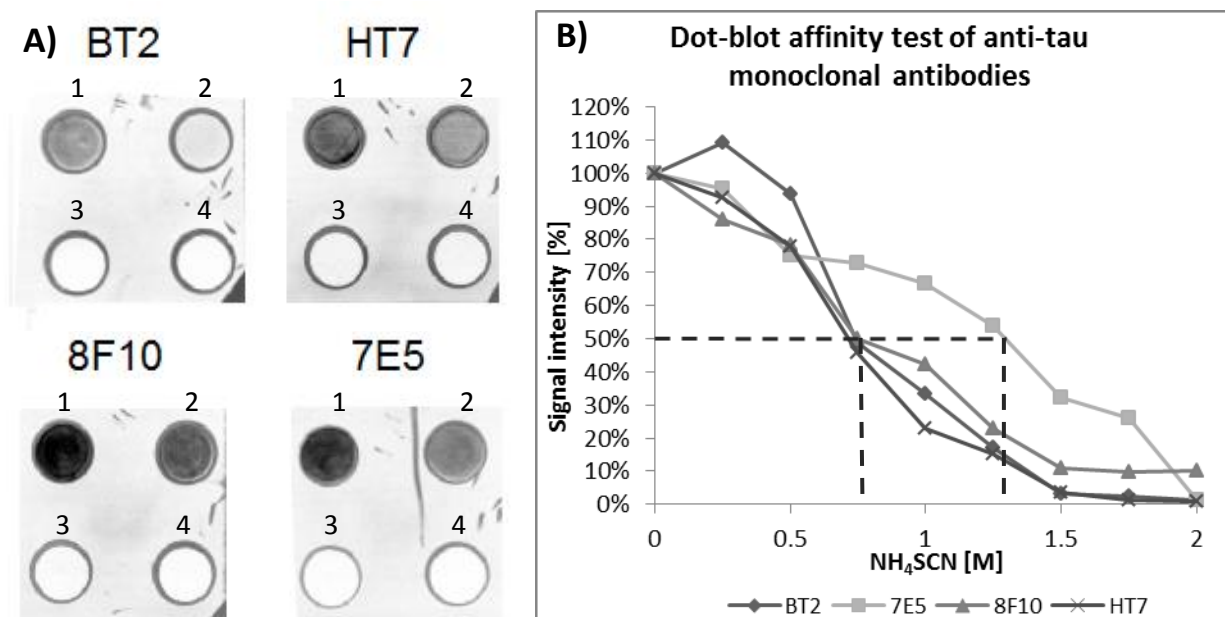


Figure 3: Dot-blot analysis: A – immunoreactivity of monoclonal anti-tau protein antibodies (clones HT7, BT2, 8F10 and 7E5) with recombinant tau protein isoform 441, list of samples and controls spotted on the PVDF membrane: 1 – recombinant tau protein isoform 441 (0.05 μg); 2 – recombinant tau protein isoform 441 (0.025 μg), 3 – bovine serum albumin (1 μg), 4 – phosphate buffer; B – results of dot-blot affinity test determining affinity index of monoclonal anti-tau protein antibodies (clones HT7, BT2, 8F10 and 7E5), 0.03 μg of recombinant tau protein isoform 441 was applied in triplicate for each concentration of NH_4SCN (average values are in the graph); primary antibody: HT7, BT2, 8F10, 7E5, 0.1 $\mu\text{g}/\text{mL}$; secondary antibody: anti-mouse IgG/HRP 1:1000; detection: Opti-4CN kit.

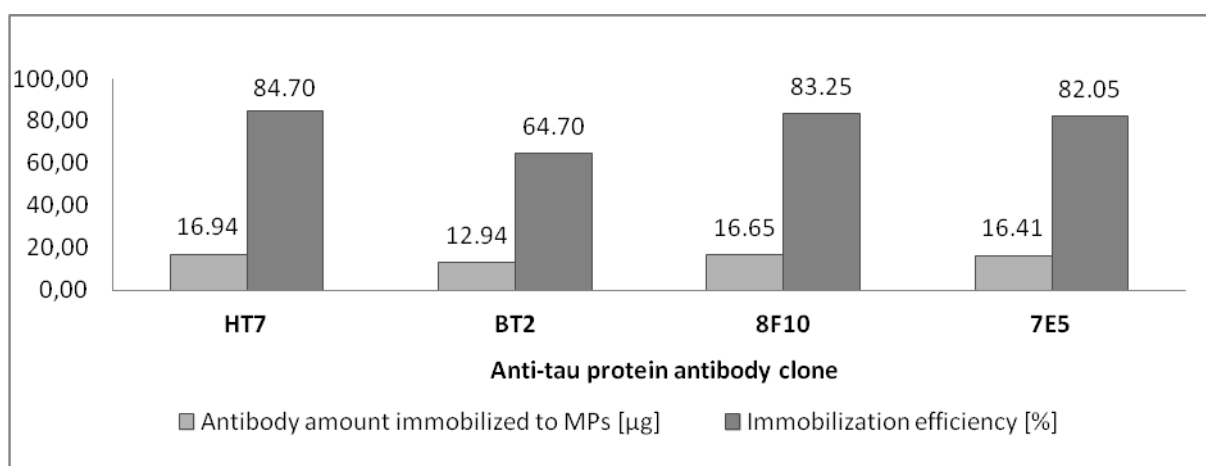
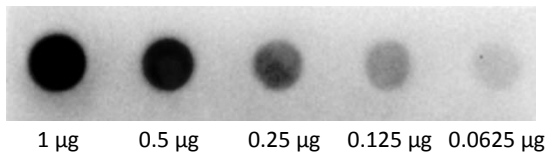
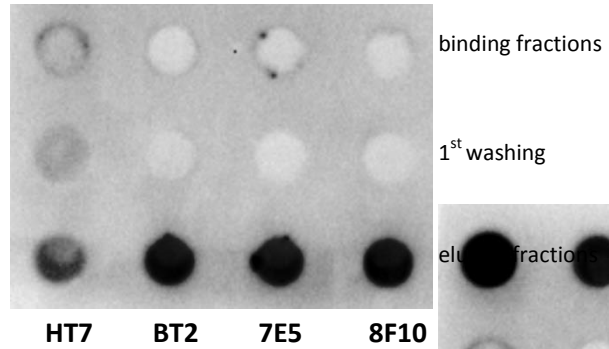


Figure 4: Binding efficiency of monoclonal anti-tau antibodies immobilized to the SeraMag MPs, protein content was determined in solution before and after the IgG immobilization by BCA kit, absorbance at 570 nm was measured.

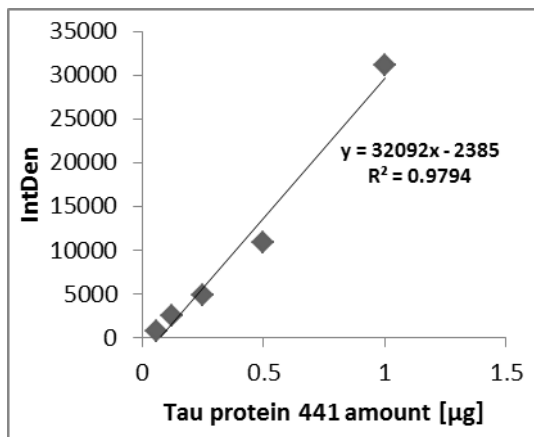
A) Dot-blot calibration



B) Dot-blot sample analysis



C) Calibration curve



D) Semi-quantification

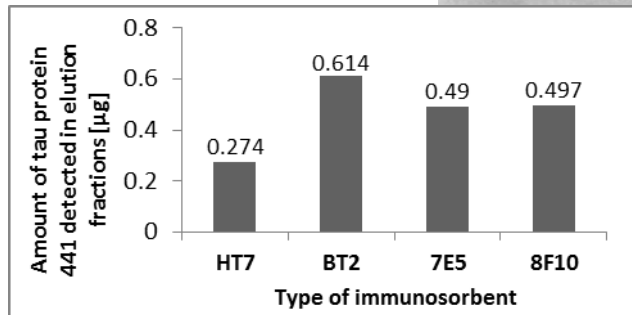
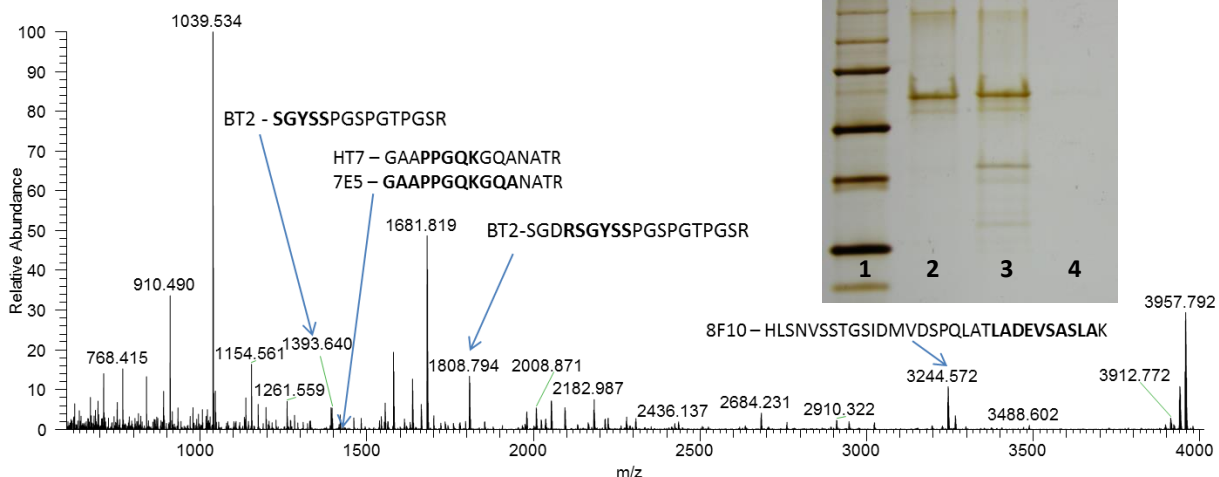


Figure 5: Determination of binding capacity of anti-tau protein immunosorbents: A – dot-blot analysis of different amounts of recombinant tau protein 441; B – dot-blot analysis of fractions collected during an immunomagnetic separation of recombinant tau protein 441; C – calibration curve constructed for semi-quantification of tau protein amount in samples; D – amount of tau protein calculated in elution fractions; primary antibody: Tau 5, 1:30000; secondary antibody: anti-mouse IgG/HRP 1:10000; detection: Clarity western ECL substrate.

A)



B)

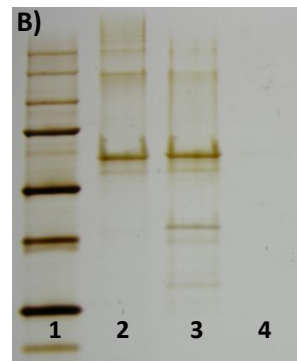


Figure 6: Analysis of recombinant tau protein 441 digestion using immobilized TPCK-trypsin: A – MS analysis; B - Tris-tricine-SDS-PAGE followed by silver staining, lanes: 1 – marker of molecular weights (10-250 kDa), 2 – recombinant tau protein 441, 3 – unfolded recombinant tau protein 441 using DTT and IAA, 4 – unfolded tau protein after digestion with immobilized trypsin.

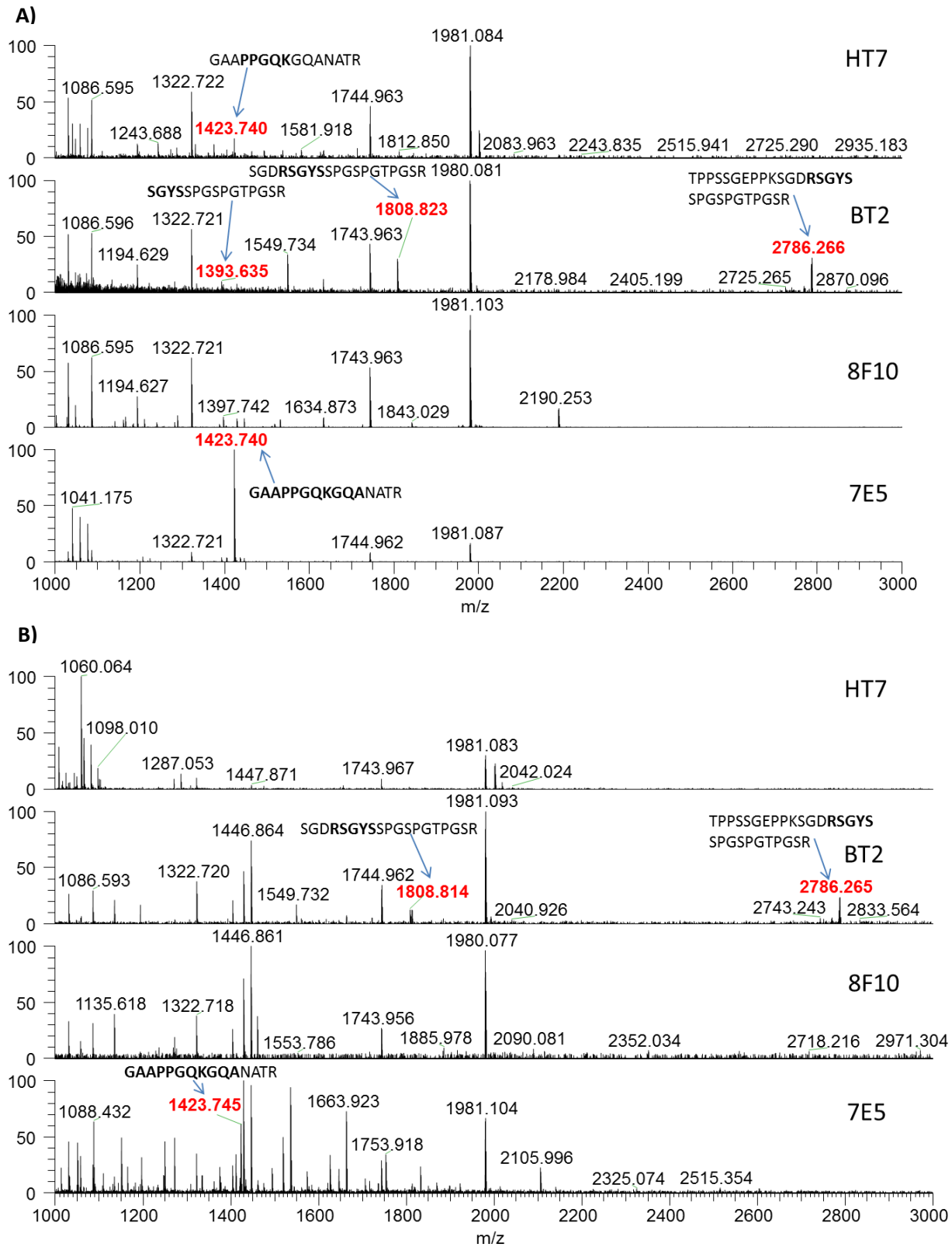


Figure 7: MS analysis of elution fractions collected within IMS using four different anti-tau immunosorbents: A – without 8M urea incubation step, B – with 8M urea incubation step; peptides containing a specific epitope sequence are highlighted in red.

Table 1: Characteristics of tested monoclonal anti-human tau antibodies.

Clone	Isotype	Antigen	Declared purity	Epitope*	Supplier
HT7	Mouse IgG1κ	Human tau	>95% (SDS-PAGE), PBS	PPGQK (aa 159-163)	Pierce
BT2	Mouse IgG1κ	Bovine tau	>95% (SDS-PAGE), PBS	RSGYS (aa 194-198)	Pierce
8F10	Mouse IgG1κ	Recombinant human tau 441	>95% (SDS-PAGE), PBS pH 7.4	LADEVASLA (aa 428-437)	AJ Roboscreen
7E5	Mouse IgG3κ	Human tau 441	>95% (SDS-PAGE), carbonate buffer pH 9.6	RGAAPPQKQQA (aa 156-165)	AJ Roboscreen

*numbering according to human Tau isoform 441; aa – amino acid residues

Table 2: List of tryptic fragments of tau protein 441 including highlighted epitopes of tested antibodies, generated by PeptideMass tool, set parameters - maximum number of missed cleavages (MC): 2, all cysteines have been treated with iodoacetic acid to form carboxymethyl-cysteine (Cys_{CM}), methionines have been oxidized to form methionine sulfoxide (MSO), displaying peptides with a mass bigger than 500 Dalton, using monoisotopic masses of the occurring amino acid residues and giving peptide masses as [M+H]⁺.

Antibody clone	[M+H] ⁺	Position	MC	Sequence
HT7	1423.7400	156-170	1	GAAPPQKQGQANATR
	725.3940	156-163	0	GAAPPQK
BT2	1808.8158	191-209	1	SGDRSGYSSPGSPGTPGSR
	434.1994	191-194	0	SGDR
	1393.6342	195-209	0	SGYSSPGSPGTPGSR
8F10	3541.7690	407-441	1	HLSNVSTGSIDMVDSPQLATLADEVASLAKQGL
	3243.6049	407-438	0	HLSNVSTGSIDMVDSPQLATLADEVASLAK
7E5	1962.0627	151-170	2	IATPRGAAPPQKQGQANATR
	1263.7167	151-163	1	IATPRGAAPPQK
	1423.7400	156-170	1	GAAPPQKQGQANATR
	557.3405	151-155	0	IATPR
	725.3940	156-163	0	GAAPPQK
	717.3638	164-170	0	GQANATR

Table 3: Summary of achieved results.

Anti-tau mAb	HT7	BT2	8F10	7E5
Purity	pure, homogeneous and non-fragmented	pure, homogeneous and non-fragmented	pure, homogeneous and non-fragmented	pure, homogeneous and non-fragmented
Affinity index	0.75 mol/L (+)	0.75 mol/L (+)	0.75 mol/L (+)	1.3 mol/L (++)
Specific reactivity	strong	weaker (+)	strong	strong
Cross-reactivity	-	-	-	-
Immobilization efficiency	84.7 %	64.7 %	83.25 %	82.05 %
Binding capacity (μg of tau/0.5 mg of IS)	0.274 μg	0.614 μg	0.497 μg	0.49 μg
Peptide reactivity (without urea)	+	+	-	++
Peptide reactivity (without urea)	-	+	-	+